

Beyond the DNA: A Prototype for Functional Genomics

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Beyond the DNA: A Prototype for Functional Genomics

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Introduction:

A prototype oligonucleotide "functional chip" has been developed to screen novel DNA repair proteins for their ability to bind or alter different forms of DNA. This chip has been developed as a functional genomics screen for analysis of protein-DNA interactions for novel proteins identified from the Human Genome Project. The process of novel gene identification that has ensued as a consequence of available sequence information is remarkable. The challenge now lies in determining the function of newly identified gene products in a time- and cost-effective high-throughput manner. The functional chip is generated by the robotic application of DNA spotted in a microarray format onto a glass slide. Individual proteins are then analyzed against the different forms of DNA bound to the slide. Several prototype functional chips were designed to contain various DNA fragments tethered to a glass slide for analysis of protein-DNA binding or enzymatic activity of known proteins. The technology has been developed to screen novel, putative DNA repair proteins for their ability to bind various types of DNA alone and in concert with protein partners. An additional scheme has been devised to screen putative repair enzymes for their ability to process different types of DNA molecules.

Current methods to analyze gene expression primarily utilize either of two technologies. The oligonucleotide chip, pioneered by Fodor and co-workers and Affymetrix, Inc., consists of greater than 64,000 oligonucleotides attached *in situ* to a glass support (Fodor et al., 1991; Affymetrix, Inc; Santa Clara, CA). The oligonucleotide chip has been used primarily to identify specific mutations in a given gene by hybridization against a fluorescently-labeled substrate. The second method is the microarray, whereby DNA targets are systematically arranged on a glass slide and then hybridized with fluorescently-labeled complex targets for gene expression analysis (Jordan, 1998). By this technique, a large amount of information can be obtained examining global differences in gene expression among different cell populations, disease states and following drug treatments or toxic insult to whole cells. However, these technologies have yet to be extended beyond the examination of DNA/RNA molecules to dissect gene function.

One of the most critical components of cellular biochemistry is the integrity of interaction between proteins and DNA. Protein-nucleic acid interactions are essential for the structural organization, replication, repair and expression of genetic information. Understanding the complexities of protein-DNA interactions is a fundamental step towards comprehending key aspects of disease biochemistry. The complexity of these

cellular processes generally makes it necessary to analyze these interactions *in vitro*. Many techniques have been designed to offer insight into these processes including filter binding studies, electrophoretic mobility shift assays and footprinting technologies. But these techniques are laborious and time-consuming, and the development of high-throughput strategies for analysis of protein-DNA interactions would greatly propel these areas of research. Using an oligonucleotide chip approach, novel proteins can be analyzed for DNA binding or enzymatic characteristics. Proteins can be screened against the "functional chip" both individually and in combination.

Methods:

Synthesis of DNA substrates and application to chips.

The "functional chip" was designed to contain different types of DNA substrates (oligonucleotides initially up to 50 nucleotides in length) to be analyzed against recombinant proteins individually and in combination to determine whether the proteins bind DNA. Precleaned microscope slides were prepared for attachment of oligonucleotides using a modified protocol of Guo et al., 1994. Slides were then activated with the addition of a linker molecule to allow binding of the DNA to the glass surface (unpublished protocol courtesy of A. J. Wyrobek, LLNL). Specially designed oligonucleotides were synthesized and PAGE-purified using standard phosphor-amidite chemistry (Operon Technologies Inc.; Alameda, CA). Double-stranded DNA was generated by the hybridization of homologous oligonucleotides. One complementary oligonucleotide was employed for generation of double-stranded substrates. The oligonucleotides were applied to the slide in a microarray format using a Newport spotter (Norgren Systems; Palo Alto, CA) at 25 pmol/ μ l in NaCO_3 buffer, pH 8. Subsequently, slides were blocked with 1 % ammonium hydroxide solution for 30 minutes to prevent non-specific binding. The spotted slides can be stored indefinitely under nitrogen at -20°C.

Use of robotic spotting greatly facilitated the production of microarrays for these experiments. Initial analysis examined the spotted DNA on the slide visually by application of the general DNA stain Yo-Yo (Molecular Probes; Eugene, OR). In addition, the oligonucleotides were designed to contain a restriction enzyme site to confirm the integrity of the double-stranded DNA by restriction enzyme digestion following the attachment to the glass.

Application of proteins to "functional chip": DNA binding assay.

Initial experiments examined proteins known to bind DNA, such as RecA and tubulin, to characterize DNA binding parameters on the newly designed functional chip. Slides bound with oligonucleotides were incubated in binding buffer (100 mM Tris pH

7.5, 0.6 M NaCl, 0.4 M MgCl₂, 1 mg/ml BSA, 1 M DTT) at room temperature for 15 minutes to prepare the slide for protein binding. Individual recombinant proteins (RecA: New England Biolabs; Beverly, MA; tubulin, actin: Sigma Chemicals, Inc., St. Louis, MO; APE proteins were kindly provided by Dr. David Wilson, BBRP) were applied to the slide in binding buffer for 15 minutes. Unbound protein was removed by serial washing in binding buffer. The protein-DNA interaction was crosslinked in 3% glutaraldehyde/TBS (50 mM Tris, 150 mM NaCl) and the excess glutaraldehyde immediately removed. Non-specific binding was blocked by the application of swine antiserum (Dako; Carpinteria, CA). Protein-specific antibody (RecA: Medical & Biological Laboratories; Nagoya, Japan; tubulin, actin: Sigma Chemicals, Inc., St. Louis, MO; APE antibody was kindly provided by Dr. David Wilson, BBRP) was applied to the slide and incubated overnight at 4°C. Excess antibody was removed by washing and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Boehringer Mannheim; Indianapolis, IN) was applied for 1 hour. Unbound secondary antibody was removed and Vectorshield (Vector; Burlingame, CA) was applied to prevent quenching of the fluorescent signal.

Application of proteins to “functional chip”: Enzymatic assay.

Prototype experiments examined the apurinic endonuclease protein, APE, known to excise DNA, to characterize the utility of the functional chip for enzymatic analysis. Slides bound with oligonucleotides were incubated in binding buffer (50 mM HEPES-KOH pH 7.5, 50 mM KCl, 100 µg/ml BSA, 10 mM MgCl₂, 0.05% Triton X-100) for 1 hour at room temperature. Following this incubation, excess protein was removed by washing in PN buffer (20 mM NaH₂PO₄, 100 mM Na₂HPO₄). Slides were incubated in Texas-Red labeled avidin (Vector; Burlingame, CA) in PNM buffer (5% milk in PN buffer, 0.02% Na Azide) for 20 minutes. Excess avidin was removed by washing in PN buffer and the slides were incubated in biotin-labeled anti-avidin antibody (Vector; Burlingame, CA). Excess antibody was removed and slides were incubated in Texas-Red labeled biotin. Vectorshield (Vector; Burlingame, CA) was applied and slides were visualized. The removal of APE-incised DNA was performed as follows. The Vectorshield was removed by washing and the slide was heated to 80°C for 20 minutes, incubated in Texas-Red labeled avidin for twenty minutes and excess avidin was removed by washing. Slides were next incubated with biotin-labeled anti-avidin antibody for 20 minutes followed by incubation with the Texas-Red labeled avidin. All incubations were performed at room temperature. Vectorshield was applied and the slides were re-visualized.

Imaging of the functional chip.

Images of the slides were digitally acquired using a full-field imaging system from Norgren Associates (Palo Alto, CA). This system was used to detect and quantify the fluorescent signals on the slide after the immunocytochemical applications were completed. Results were analyzed using an UV microarray reader (Norgren Systems; Palo Alto, CA) with a FITC filter set. Images were captured using Image Workstation software version 0.01 (Norgren Systems; Palo Alto, CA).

Results & Discussion:

The DNA substrates used in the design of the functional chip were short oligonucleotides and were readily synthesized commercially. A modified amino-terminal group was used to chemically attach the DNA to a standard glass microscope slide. The types of oligonucleotides included on the chip were designed with relevance to the putative functional pathways in which the proteins initially examined are implicated to participate. In preliminary experiments, we have demonstrated the DNA binding properties of RecA, a recombinational repair protein from *E. coli* (Roca and Cox, 1997), and tubulin and actin, cytoskeletal proteins, (Olmstead, 1986; Pollard and Cooper, 1986) on the functional chip. Additional experiments were conducted to determine detectable levels of signal from known protein concentrations. Using a prototype chip, we have also demonstrated the utility of the chip for enzymatic analysis using the human apurinic endonuclease, APE (Wilson III et al., 1997). APE specifically incises the DNA backbone of double-stranded DNA that contains an apurinic site (Wilson III et al., 1997). As shown in Figure 1, we demonstrated the specific enzymatic activity of APE on the functional chip. A chip was fabricated containing three rows of oligonucleotides: one row of double-stranded DNA with a biotin-labeled oligonucleotide and its complimentary strand, another row of the same double-stranded oligonucleotide with an apurinic site, and a third row of single-stranded oligonucleotide containing an apurinic site and a biotin label. APE was applied to the slide and the DNA was visualized to detect the biotin on the DNA. Upon heating to 80°C, the double-stranded DNA denatured to single-stranded DNA by the breaking of hydrogen bonds between the strands. This allowed for the double-stranded DNA containing the apurinic site that was cut with APE to be removed, leaving behind only single-stranded DNA up to the apurinic site thereby effectively removing the biotin label. After washing the slide and reincubation with the anti-biotin antibody and fluorescently-labeled secondary, no biotin label was detected on the double-stranded DNA containing an apurinic site demonstrating the incision of the DNA by the APE enzyme.

Future chips can be tailored to include any of several types of double-stranded DNA, single-stranded DNA and structurally-modified DNA which are present or formed

during the course of different types of DNA repair. In addition, several structurally modified double-stranded DNA can be devised to contain a 5' end overhang or a 3' end overhang, or a more complex structure like that of a Holliday junction (West, 1998) can be incorporated into the formation of the double-stranded sequences. Up to twenty different DNA types will be represented in the design of the final DNA repair functional chip.

Purified protein was applied individually to the slide - one protein, one slide. However, the microarray affords the potential to analyze multiple proteins (3 or 4) on the same DNA pools on one slide. This will be investigated in future experiments. As protein-DNA interactions are unique to each protein involved, a matrix of conditions needs to be examined to test for DNA binding or enzymatic activity (buffer composition, time, protein concentration, and temperature). Both time course experiments as well as concentration-dependent experiments will be performed.

Using a newly developed, automatable high-throughput protein expression system, selected cDNAs contained within the I.M.A.G.E. Consortium cDNA collection (Lennon et al., 1996) can be used in a baculoviral expression paradigm for the generation of recombinant proteins for analysis on the functional chip. This presents a novel method for the expression and examination of proteins from the largest, publically available database of cDNA libraries. The advantage of this approach is the ability to access novel repair proteins within the Human Genome by the expression and analysis of any representative gene. Future work will focus on the generation of the components of the recombinational repair pathway for functional analysis using this novel chip technology. Initially, full-length proteins will be used against the functional chip to determine binding characteristics. Proteins will then be examined in more detail by the use of partial protein fragments expressed and purified to identify DNA binding or enzymatic domain(s). The chip can also be used to identify functional mutants in these repair proteins. This is the first demonstration of functional activity in a microarray format that is amenable to high-throughput screening using the resources of genome technologies. Future work with the functional chip will aim to facilitate the identification of novel DNA repair proteins.

Figure 1: Ape incises DNA on the Functional Chip.

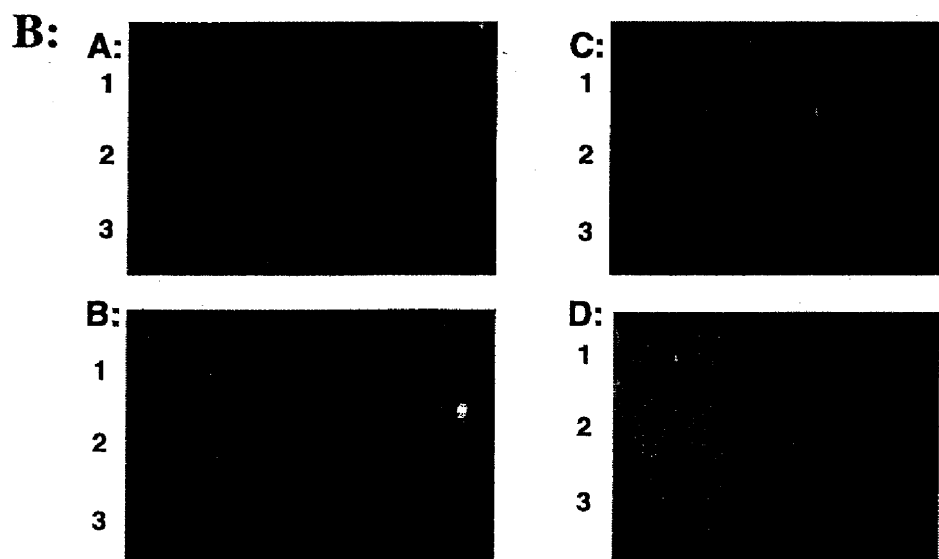
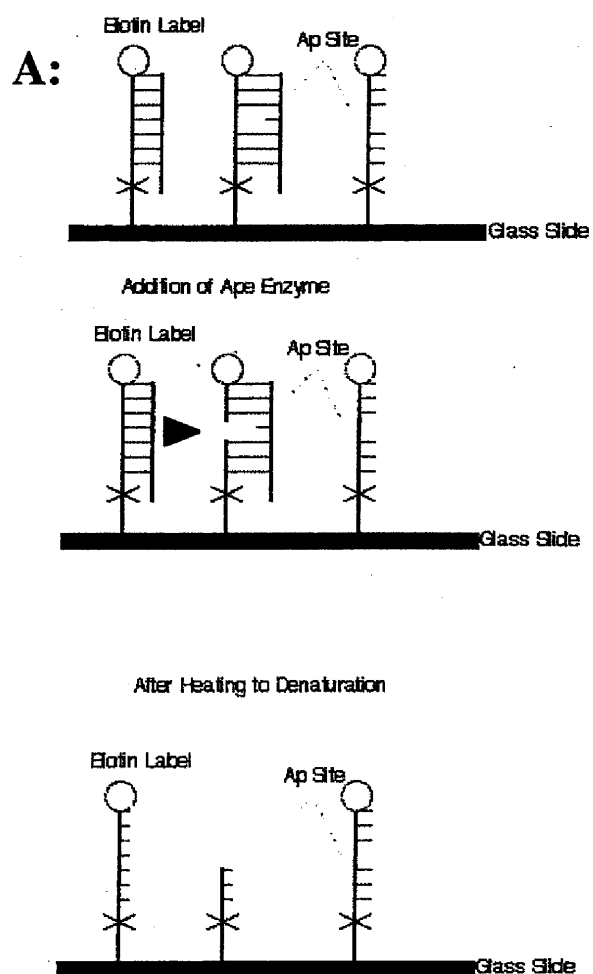


Figure Legend:

Panel A: This cartoon illustrates the oligonucleotides represented on the functional chip incubated with the APE protein. The top schematic shows the DNA components attached to the glass slide: double-stranded DNA containing a biotin label, double-stranded DNA containing an abasic site (AP) and a biotin label, and single-stranded DNA containing an abasic site and biotin label. The middle schematic shows the condition of the DNA after incubation with APE before heat-treatment. The arrowhead indicates where the DNA backbone has been incised. The bottom schematic represents the removal of incised DNA after incubation with APE and heat-treatment. Heat denatures the DNA liberating the complementary and excised region of DNA containing the biotin label.

Panel B: APE specifically incises the DNA backbone of double-stranded DNA that contains an apurinic site. Subpanels A-D: Row 1 - double-stranded DNA with biotin label. Row 2 - double-stranded DNA with an apurinic site and biotin label. Row 3 - single-stranded DNA with apurinic site and biotin label. Panel A: Incubation and visualization with APE before heat denaturation. Panel B: Incubation and visualization with APE after heat denaturation. Panel C: Buffer only before heat treatment. Panel D: Buffer only after heat treatment. All slides were incubated with a fluorescently-labeled secondary antibody for visualization. Heat denaturation and re-visualization of the functional chip after the addition of the APE enzyme showed no detection of the biotin label. After viewing the slide for the second time, enzymatic activity was detected only on double-stranded DNA containing an apurinic site (Panel B, Row 2).

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